Gene electrotransfer: from biophysical mechanisms to in vivo applications

Part 1- Biophysical mechanisms

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Abstract Electropulsation is one of the nonviral methods successfully used to deliver genes into living cells in vitro and in vivo. This approach shows promise in the field of gene and cellular therapies. The present review focuses on the processes supporting gene electrotransfer in vitro. In the first part, we will report the events occurring before, during, and after pulse application in the specific field of plasmid DNA electrotransfer at the cell level. A critical discussion of the present theoretical considerations about membrane electroporation and the transient structures involved in the plasmid uptake follows in a second part.

Keywords Electroporation · Electroporation · Gene electrotransfer · Biophysical mechanisms · Membranes

Introduction

Electroporation results from a controlled application of electric pulses to cells, leading to their transient and

reversible membrane permeabilization (Neumann et al. 1989; Teissié et al. 2005; Escoffre et al. 2007). This process brings new properties to the cell plasma membrane, which, besides being permeabilized, becomes fusogenic and allows exogenous proteins to be inserted in it. Electroporation is used to introduce a large variety of molecules into many different cells in vitro (Orlowski and Mir 1993; Eynard et al. 1997). Clinical applications of the method are now under development as results of the EU Cliniporator and ESOP programs. A local anti-tumoral drug delivery to patients (a method called electrochemotherapy) is in clinical trials in tens of hospitals through Europe (Mir et al. 1995; Cemazar and Sersa, 2007; Sersa et al. 2008; Mir et al. 1998, 2006). The most frequent application of electric field-induced membrane permeabilization is the transfer and expression of genes into mammalian cells. However, this application not only involves the introduction of DNA into cells, but also depends on subsequent cellular processes (Weaver and Chizmadzhev 1996). The transfer of naked DNA plasmid and the expression of the gene of interest are enhanced by electroporation into different tissues, including the skeletal muscle (Aihara and Miyazaki 1998; Mir et al. 1999), liver (Heller et al. 1996; Liu and Huang 2002), skin (Titomirov et al. 1991; Vandermeulen et al. 2007), and tumors (Rols et al. 1998a). The transfection efficiency of this physical method in vivo is still low compared to the viral vectors. However, due to the ease with which it is performed and its speed, reproducibility, and safety, gene electrotransfer holds a great potential for clinical application.

One of main limits of the widespread use of electroporation is that very little is known about the biophysical mechanisms supporting the reorganization of the cell membrane (pore, electropore, defects?). The

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molecular target of the field effect remains unclear (Teissié et al. 2005). The other main limit in gene electrotransfer comes from the transfer inside the nucleus. In nondividing cells, the nuclear envelope is an especially problematic hurdle to gene transfer, which should take place through the nuclear pore complex (Van der Aa et al. 2006, 2007; Pouton et al. 2007). No direct biophysical method to alter the nuclear envelope or pore has been reported (yet). A successful approach is in modifying plasmid (pDNA) vectors to enhance nuclear import through the NPC (Miller and Dean 2009).

The present review focuses on the processes supporting gene electrotransfer *in vitro*. The events occurring before, during, and after pulse application leading to gene electrotransfer will be described. Theoretical considerations about membrane structures involved in the plasmid uptake will be described in a (very) critical manner. In a companion paper, *in vivo* gene electrotransfer and its clinical applications will be addressed.

Biophysical mechanisms

In vitro biophysical considerations

Gene electrotransfer to mammalian cells is obtained by mixing cells and plasmids in a biocompatible buffer (control of pH, osmolarity, conductivity), then by applying a well-controlled electric field pulse train (shape of pulses, choice of field strength, pulse duration, number of pulses, delay between pulses) and finally bringing the mixture into a culture medium. Expression of the coded activity can be detected after a couple of hours but reaches a high level after 24 h (Golzio et al. 2004). Transfer of a large polyelectrolyte across the membrane requires a transient loss of cohesion of the proteolipidic assembly. This dramatic event should nevertheless preserve the cell viability to observe the expression of the activity coded by the plasmid.

Experimental facts: events before, during, and after electropulsation

The plasma membrane is not permeable to hydrophilic molecules such as nucleic acids. Plasmid molecules, due to their negative charge, cannot interact with the cell plasma membrane that bears the same charge. DNA molecules therefore cannot get access to the cytoplasm. No transfected cells are detected in the absence of plasmid or when plasmid is added after the electropulsation. The prepulse incubation time is not very important but to avoid a negative effect of exogenous nucleases should be kept short and processed at a low temperature (Rols et al. 1994).

Indeed, gene expression is obtained after applying electric pulses to plasmid/cell mixture after a short incubation (Neumann et al. 1982; Klenchin et al. 1991; Wolf et al. 1994). These results are the same whatever the cell types (bacteria, yeast, and mammalian cells) (Golzio et al. 1998; Eynard et al. 1992; Ganeva et al. 1995). Preadsorption of DNA on the mammalian cell surface is not requested and was indeed reported to prevent expression (Neumann et al. 1982).

Gene electrotransfer is only detected for electric field values leading to membrane permeabilization ($E_c > E_p$). Transfection threshold values are the same as the ones for cell permeabilization when millisecond pulses are applied (Rols and Teissié 1998). Field strength is observed to have a critical role. Plasmid molecules, negatively charged, migrate when submitted to an electric field (Wolf et al. 1994; Neumann et al. 1992). In the “low” electric field regime (i.e., $E_c < E_p$), plasmid simply electrophoretically flows along the cell membrane towards the anode. It can be trapped when divalent ions are present (Francescu et al. 2005; Xie and Tsong 1993).

However, beyond a critical permeabilizing field value ($E_c > E_p$), two main processes occur: (1) plasma membrane is permeabilized (at the microsecond scale); (2) plasmid undergoes the electrophoretic migration (at the millisecond scale) and interacts with permeabilized membrane (Golzio et al. 2002). Metastable plasmids/membrane complexes are formed and grow as local aggregates (at the millisecond scale). But once the field is turned off, the growth of the plasmid aggregates is stopped. The plasmid/membrane interaction is not homogeneously distributed on the permeabilized areas but is detected in association with membrane competent-like sites whose sizes range from 0.1 to 0.5 μm . This interaction takes place only when the plasma membrane is under permeabilization (a field over E_c must be present) (Golzio et al. 2002). It does not occur if the plasmid molecules are added after electroporeabilizing cells (Neumann et al. 1982; Tsong 1991; Klenchin et al. 1991). Recent works on electric field vectoriality show that the plasmid/membrane interaction is not a simple accumulation of plasmid at the membrane surface level, but a plasmid “irreversible” insertion occurs into the permeabilized membrane. No free plasmid diffusion into the cytoplasm is detected as was proposed in older works (Klenchin et al. 1991).

Under permeabilizing field conditions, the pulse duration plays a critical role in the formation of the plasmid/membrane complexes. These complexes are easily detected when the pulse duration is at least 1 ms, but highly sensitive technologies show that they are formed with shorter pulses (unpublished data). This suggests that the density of defects is critical in the plasmid/membrane interaction. Furthermore, this interpretation is supported by the observation

that the plasmid content in the complex is under the control of the field strength (E), the number of successive pulses (N), and the pulse duration (T) (Golzio et al. 2002). The time for reaction with the membrane of the plasmid dragged against permeabilized membrane under the electrophoretic migration is increased by long pulse durations. This again is involved in the positive role of the pulse duration in gene electrotransfer. This contribution of the pulse duration to the plasmid/membrane interaction has already been illustrated by a complex dependence of the gene expression (Wolf et al. 1994). The associated gene expression Expr is shown to obey the following equation:

$$Expr = KNT^{2.3} \left(1 - \frac{E_p}{E} \right) f(DNA)$$

as long as the cell viability is not affected to a large extent by the pulse duration (Golzio et al. 2002). All parameters are as described above, with K being a constant and N , the number of electric pulses. Its dependence on the plasmid concentration is rather complex, as high levels of plasmids appear to be toxic (Rols et al. 1992). The practical conclusion is that in vitro an effective transfer is obtained by using long pulses in order to drive the plasmid towards the permeabilized membrane but with low field strength to preserve the cell viability (Rols and Teissie 1998; Kubiniec et al. 1990). Nevertheless, the gene transfection was obtained with short long pulses in the pioneering experiments (Neumann et al. 1982), in a very recent report (Kanduser et al. 2009), and with stem cells (Ferreira et al. 2008).

Plasmid/membrane complex remains accessible to DNase I, up to 60 s after the pulsation in the case of CHO cells. The plasmid aggregates, which are anchored in the membrane after the electric field application, remain sensitive to the degrading action of the nucleases that were added externally post-pulse, which are known not to cross the membrane (Eynard et al. 1997). Nevertheless, the opposite observation, reported as a protective effect against DNase, was detected when using a different protocol (Klenchin et al. 1991). More than 2 s appears to be needed to get a stable plasmid/membrane complex after a 5 ms pulse (Phez et al. 2005). Several minutes after the electropulsation, plasmids are still present on/in the cell surface. The biophysical structure of the membrane plasmid complex has to be explained. Plasmids leave the complex and diffuse in the cytoplasm. Then, plasmids are present at the nucleus surface, but only a small fraction cross the nuclear envelope to be expressed a few hours after the electropulsation (Golzio et al. 2002). These intracellular steps remain rather poorly understood as already mentioned. A positive effect of working at 37°C in all these post-pulse steps was observed (Rols et al. 1994). Hypo-osmolar buffers gave an increase in expression (Golzio et al. 1998). Starving cells prevented expression, suggesting

an active contribution of the cell metabolism in transfer and/or expression (Rols et al. 1998b) (Table 1).

Theories of DNA plasmid electroentry

Although the first pioneering report on gene electrotransfer in cells was published more than 25 years ago by E. Neumann, the molecular basis behind the process of gene electrotransfer is highly debated. Different scenarios have been proposed (Fig. 1):

- (i) Krassowska's model supports the simplest mechanism, in which plasmid enters the 5 nm thick membrane through stable macropores (i.e., 20 to 200 nm Ø) (Smith et al. 2004; Krassowska and Filev 2007). The electrically induced defects result from the field-associated membrane potential changes. Their model relies on tension-coupled pores that do not bring membrane rupture [a dramatic feature of the classical Chizmadzhev model (Weaver and Chizmadzhev 1996)]. It predicts a post-pulse growth of macropores on the seconds time scale fairly consistent with experimental evidence (Neu and Krassowska 2003). This model predicts pores large enough to permit the plasmid uptake, even in its circular or supercoiled conformation (Blackburn and Gait 1996). These pores remain open for the entire duration of electropulsation providing adequate time for the plasmid to enter the cell (Sukharev et al. 1994).

Table 1 Steps in gene electrotransfer. The central column describes the events that occur before, during, and after the electropulsation. The right column reports their time scale

Steps	Events	Time scale
Before electropulsation	Plasma membrane is not permeable. Plasmid cannot get access to the cytoplasm.	
During electropulsation	Electropermeabilization takes place.	Microseconds
	Plasmid molecules are electrophoretically driven into contact with the cell surface.	Milliseconds
	Metastable complexes are formed between plasmid molecules and the localized electropermeabilized part of cell membrane.	Milliseconds
After electropulsation	Stable complexes result.	Seconds
	Plasmids leave the complexes and diffuse in the cytoplasm.	Minutes
	A small fraction of the plasmid molecules cross the nuclear envelope to be expressed.	Hours
	Gene expression occurs.	Days

Fig. 1 Different models of gene electrotransfer. *Model i*: *a* The electric pulse induces a macrodefect ($2r > 20$ nm) and an electrophoretically mediated DNA accumulation. *b* After the pulse, a free diffusion of DNA takes place across the long-lived macropore. *Model ii*: *a* DNA is preadsorbed with the plasma membrane by an interaction with cationic lipids, sphingosines. *b* DNA is transiently inserted in plasma membrane. *c* DNA is electrophoretically pulled through the permeabilized zones. *d* DNA leaves the membrane and enters into the cell. *Model iii*: *a* The electric pulses induce the membrane permeabilization. *b* DNA is concentrated near the membrane surface and

pushed through the putative electropores by electrophoretic forces. *c* The mechanical interaction between the pores and the DNA driven by electrophoretic forces induces an adjustment of pore sizes. *c* DNA enters into the cell. *Model iv*: *a* DNA remains at the interfacial region when no pore is present. *b* Under high electric field, the DNA diffuses towards the interior of the bilayer after a pore is created beneath it. *c* Diffusion of the strand toward the interior of the membrane leads to a complex DNA/lipid in which the lipid head groups encapsulate the DNA. *d* After the electric field pulse, the DNA is translocated. *e* DNA enters into the cell

This model remains an attractive model in spite of the existence of many experimental contradictions. Indeed, until now, no study made it possible to visualize these membrane pores. This validation appears impossible (Weaver and Chizmadzhev 1996). Moreover, the resealing time of pores appears to be shorter in this model than in experiments (e.g., seconds rather than minutes) (Bier et al. 1999; Golzio et al. 1998; Satkauskas et al. 2002; Tekle et al. 1991). In addition, in the case of CHO cells, plasmid accessibility to DNAase I in the minute following the end of electropulsation shows that the plasmid transfer inside the cell occurs after the electropulsation (Eynard et al. 1997). Of course, no selectivity of the macropore is

predicted meaning that DNAases have free access. To date, theoretical models could predict stable pores of only a few nanometers in radius; larger pores are unstable (Freeman et al. 1994; Joshi and Schoenbach 2000). These models are confirmed by some experiments in which high-voltage, short pulses are used that must have created a large number of pores with radii of about 1 nm (Glaser et al. 1988; Kakorin and Neumann 2002; Schwister and Deuticke 1985). To reconcile these results with the experimental evidence of plasmid translocation after electropulsation, some researchers postulated that plasmid entry into cells relies on the plasmid/membrane interactions, which may be facilitated by a coalescence of many small, 1 nm pores

(Neumann et al. 1996; Rols and Teissié 1998; Rols et al 1998b; Sukharev et al 1992, 1994). The slow transport of DNA across the electroporabilized membrane reflects a highly interactive electrotransfer, where many small pores coalesced into large pores that were transiently occluded by DNA (Neumann et al. 1999).

- (ii) Other data report that gene electrotransfer through lipid bilayer could be mediated by transient complexes between plasmid and the lipids in the pore edges of elongated, electroporated hydrophilic pore zones (Hristova et al. 1997). This is present with specific lipids. Moreover, the plasmid association with a lipid bilayer greatly facilitates the transport of small ions. This suggests a locally conductive plasmid/lipid interaction zone where parts of the plasmid may be inserted in the bilayer, leaving other parts of the plasmid probably protruding out from the outer surface of the bilayer. Plasmid is not only transiently inserted in, but also actually electrophoretically pulled through the permeabilized zones onto the other membrane side ultimately leaving the bilayer structure basically intact (Spasova et al. 1994). With such a model, in the case of mammalian cells, the resting potential difference should be the driving force for plasmid translocation after the pulse-induced insertion. This has not been checked yet.
- (iii) Previous works suggested that electric pulses induce the membrane permeabilization, then plasmid molecules are concentrated near the membrane surface and pushed through the putative electropores by electrophoretic forces (Winterbourne et al. 1988; Klenchin et al. 1991; Sukharev et al. 1992; Tekle et al. 1994). The mechanical interaction between the pores and the plasmid driven by electrophoretic forces induces an adjustment of pore sizes and/or lifetimes to allow plasmid entry in the cell. The plasmid may interact with the electroporabilized membrane in three possible ways: (1) The plasmid coil is aligned in an electric field, and at the appropriate pulse polarity it moves toward the permeabilized membrane. The plasmid may interact with a single membrane defect; it becomes enhanced upon plasmid interaction by the action of electrophoretic forces. (2) The passage of the (linearized) plasmid coil can be initiated by penetration of one end of the thread, which then leads the whole molecule through one pore. (3) The plasmid molecule can be involved in two pores (or more) and moving with electrophoretic forces, it cuts the membrane between these pores as a sharp thread can do (Sukharev et al. 1992). Transfer is dependent on electrophoretic forces and is complete at the end of the pulse. Interestingly, hypothesis (1) suggests a

deformation of the electric field distribution close to the membrane. The large membrane defect is associated with enhanced membrane conductivity. The electric field lines will focus on the defect carrying more plasmids to it. This is in agreement with the formation of the experimentally detected plasmid aggregates (Golzio et al. 2002).

If the electrophoretic forces are the only driving forces of the plasmid transfer into the cell, similar transfection efficiencies should be obtained for equal ET values (i.e., E , field strength and T , pulse duration). However, in the case of HeLa cells, the number of transfected clones as a function of ET values is different according to whether short or long electric pulses are used (Hui 1995). Then, when the ENT value is constant, transfection rate depends preferentially on T (Rols and Teissié 1998). Therefore, the electrophoretic migration cannot be the only driving force of the plasmid transfer into the cells but clearly supports the formation of aggregates.

- (iv) A molecular dynamic approach gives a mechanism by which plasmids do not translocate across the membrane during the electropulsation (Tarek 2005). The DNA/lipid system simulation was undertaken starting from a well-equilibrated 12 bp DNA duplex placed near a model POPC bilayer. The perturbation of the system under a 1.0 V nm^{-1} transverse electric field is followed for 2 ns. Under this high electric field, the DNA duplex diffuses towards the interior of the bilayer only *after* the creation of a pore beneath it, and within the same timescale, it remains at the interfacial region when no pore is present. Diffusion of the strand toward the interior of the membrane leads to a DNA/lipid complex in which the lipid head groups encapsulate the strand. The partial charges carried by the zwitterionic phosphatidylcholine groups of the lipids are known to be efficient for neutralizing the charges carried by the DNA (Bandyopadhyay et al. 1999). Such interactions between the plasmid and the lipids contribute to the effective screening of DNA charges and therefore to the stabilization of the complex.

The process described herein provides support to the gene delivery model by Golzio and collaborators (Golzio et al. 2002), in which it is proposed that only localized parts of the cell membrane brought to the permeabilized state are competent for transfer and that the proper transfer of plasmid—that does not require that the electric pulse be maintained—is preceded by an “anchoring step,” connecting the plasmid to the permeabilized membrane, which takes place during the pulse. One should not forget that electropulsation-mediated gene delivery concerns much

larger supercoiled plasmids than the 12 bp construct considered here. Transfer of such plasmids is certainly a complex process and all aspects may not be addressed by simulations (Tarek 2005). Nevertheless this model shows that the plasmid is stabilized in the membrane core after electropulsation in agreement with the overall experimentally observed process of DNA translocation. DNA migration from the outer side of the cell membrane to the cytoplasm is beyond this simulation study, and no calculation was carried out to follow the resealing process.

Conclusions

The molecular mechanisms involved in the phenomenon of gene electrotransfer remain poorly understood. The study of the mechanisms of gene electrotransfer shows that permeabilization is necessary but that the mechanism of the transfer of the DNA molecules is different from that of the small molecules (Table 1) (Teissié et al. 2005). The current models described in this review are sometimes contradictory and do not always fit the experimental facts (Fig. 1). Indeed, experimental observations showed that the electrotransfer is a slow mechanism. This is in contradiction with the prediction of models i and iii. Models iii and iv are in agreement with a localized plasmid/membrane interaction (so-called competent membrane sites). These sites appear to be created by the electropermeabilization process by altering the field distribution (Freeman et al. 1994). This occurs through the perturbation of the transmembrane voltage by a conducting defect. A spatially inhomogeneous electric field is expected within the outer buffer near electrode defects (Pastushenko and Chizmadzhev 1982; Powell and Weaver 1986; Barnett and Weaver 1991; Weaver 1993). This brings a nonzero component of the electric field parallel to the membrane surface, i.e., the equipotentials near the membrane have significant, nonlinear gradients. For this reason, the current flowing through a membrane electrode defect not only results in a potential drop within the membrane, but also in the buffer near the defects (Weaver and Chizmadzhev 1996). Model ii suggests a putative method for transmembrane transfer and needs further investigations. One major problem with these theoretical models is that they consider only the lipid assembly description of a membrane while the presence of membrane proteins may affect the electropermeabilization of lipid bilayers by changing their mechanical properties (Troiano et al. 1999).

The knowledge and the control of the biophysical mechanisms of gene electrotransfer are necessary to evaluate their consequences in biological and physiological terms and their effectiveness for the development of clinical and biotechnological protocols.

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